Mechanistic duality of transcription factor function in phytochrome signaling

Bassem Al-Sady*†‡, Elise A. Kikis*†§, Elena Monte*†¶, and Peter H. Quail*†‖

*Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720; and †Plant Gene Expression Center, United States Department of Agriculture/Agricultural Research Service, 800 Buchanan Street, Albany, CA 94710

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The phytochrome (phy) family of sensory photoreceptors (phyA–E in *Arabidopsis*) elicit changes in gene expression after light-induced migration to the nucleus, where they interact with basic helix–loop–helix transcription factors, such as phytochrome-interacting factor 3 (PIF3). The mechanism by which PIF3 relays phy signals, both early after initial light exposure and later during long-term irradiation, is not understood. Using transgenically expressed PIF3 variants, carrying site-specific amino acid substitutions that block the protein from binding either to DNA, phyA, and/or phyB, we examined the involvement of PIF3 in early, phy-induced marker gene expression and in modulating long-term, phy-imposed inhibition of hypocotyl cell elongation under prolonged, continuous irradiation. We describe an unanticipated dual mechanism of PIF3 action that involves the temporal uncoupling of its two most central molecular functions. We find that in early signaling, PIF3 acts positively as a transcription factor, exclusively requiring its DNA-binding capacity. Contrary to previous proposals, PIF3 functions as a constitutive coactivator in this process, without the need for phy binding and subsequent phy-induced modifications. This finding implies that another factor(s) is conditionally activated by phy and functions in concert with PIF3, to induce target gene transcription. In contrast, during long-term irradiations, PIF3 acts exclusively through its phyB-interacting capacity to control hypocotyl cell elongation, independently of its ability to bind DNA. Unexpectedly, PIF3 uses this capacity to regulate phyB protein abundance (and thereby global photosensory sensitivity) to modulate this long-term response rather than participating directly in the transduction chain as a signaling intermediate.

phytochrome-interacting factor 3 | photosensory receptor | constitutive coactivator | posttranslational regulation | *ELIP2*

The phytochrome (phy) family of sensory photoreceptors (phyA–E in *Arabidopsis*) regulates plant development in response to informational light signals (1). phys exist in two interconvertible forms: the inactive Pr and the biologically active Pfr form. Absorption of red-light (R) photons by the photoreceptor, a polypeptide with a tetrapyrrole chromophore, induces a conformational change from the Pr to Pfr form (activation); absorption of far-red light (FR) by the Pfr form triggers the converse process (2). After photoactivation, phys move from the cytosol to the nucleus and induce rapid changes in target gene expression (3). Overall, ≈10% of the *Arabidopsis* genome responds to exposure to continuous red (Rc) or far-red (FRc) light (4–6).

Phytochrome-interacting factor 3 (PIF3) is the founding member of a basic helix–loop–helix (bHLH) transcription factor family (subfamily 15 in *Arabidopsis*) that has been implicated in mediating the transcriptional activation signaled by phys (7–9). PIF3 has discrete interaction surfaces both for Pfr phyA (APA domain; active phytochrome A) and Pfr phyB (APB domain; active phytochrome B) (10, 11). PIF3 comigrates with phys A, B, and D to nuclear foci after initial light exposure of the dark-grown seedling (12). Concomitantly, PIF3 is rapidly phosphorylated in a reaction that requires direct association with phyA or phyB (11) and is subsequently degraded to a low steady-state level (7, 12, 13).

Interestingly, members of the PIF3 family (PIFs) mediate two distinct types of phenotypic responses. After initial light exposure of dark-grown seedlings, each PIF differentially mediates a characteristic response. As examples, PIF3 is required for proper chloroplast development and the expression of a set of light-induced, nuclear encoded chloroplast genes (7), whereas PIF1 regulates gibberellin biosynthesis genes to repress germination before light exposure (14) and also inhibits protochlorophyllide accumulation (15). In sharp contrast, during long-term Rc, the PIFs (including PIF3, PIF4, and PIF5/PIL6), negatively regulate the same morphogenic response: hypocotyl and cotyledon growth (7, 8, 16–18). This response is primarily controlled by the phyB photoreceptor (19) and is highly sensitive to small changes in the absolute levels of phyB (20, 21). Thus, in the case of long-term irradiation, PIF proteins regulate phyB-mediated growth responses, potentially in an additive manner.

How PIF3 accomplishes regulation of a specific subset of light-responsive genes immediately after exposure to light, yet negatively regulates morphological growth responses during long-term irradiation is unknown. Two general ideas about PIF3 function have been proposed. phys may directly transfer the light signal to DNA-bound PIF3 to affect gene expression, in a reaction requiring PIF3 to bind phys and DNA simultaneously (3, 9). Alternatively, PIF3 may repress photomorphogenesis when bound to DNA; interaction with phyA and phyB would trigger PIF3 degradation and relieve repression (22). Neither idea explains how PIF3 can act as both a positive and negative regulator.

As one approach to understanding how PIF3 performs its different roles, we asked what protein functionalities were required for each task. We performed mutant rescue experiments with transgenically expressed PIF3 variants singly abrogated in their ability to bind DNA, phyA, and/or phyB, examining the ability of each variant to function in both early, phy-induced gene expression and long-term inhibition of hypocotyl cell elongation under continuous irradiation.

Results

Rapid PIF3-Dependent *ELIP2* Induction After Initial Exposure to Light Requires Association with DNA but Not phyA or phyB. To study the role of the discrete functionalities of PIF3, we required PIF3

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‡Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143.

§Present address: Department of Biochemistry, Northwestern University, 2205 Tech Drive, Evanston, IL 60208.

¶Present address: Departament de Genètica Molecular Vegetal, Consejo Superior de Investigaciones Cientificas-Institut de Recerca i Tecnologia Agroalimentàries, 08034 Barcelona, Spain.

‖To whom correspondence should be addressed. E-mail: quail@nature.berkeley.edu.

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mutants singly defective in phyA, phyB, and DNA binding. PIF3 mutants deficient in phyA (mAPA) or phyB (mAPB) binding have been described (11) and are used here. We constructed and characterized DNA-binding defective mutants (mbHLH) [see supporting information (SI) Fig. 6] in the context of both PIF3::YFP:PIF3 minigene (YFP:PIF3), and HA-tagged *PIF3* genomic locus (HA:PIF3) constructs, and generated transgenic lines expressing these proteins in a *pif3*-null mutant background, as described in *SI Text*. Fig. 1 *A* and *B* shows schematics and terminology for all of these constructs.

A subset of rapidly responding genes (within 1 h of the light signal) depend on PIF3 for their expression after exposure to R (7). Among these genes, *ELIP2* (AT4G14690) shows the most robust dependence on PIF3 for its light responsiveness (7). Here, the absence of PIF3 decreased *ELIP2* light induction ≈2- to 3-fold at 1 h Rc (Fig. 1*C*; for quantification, see SI Fig. 9*A*). We tested whether DNA-binding, phy-binding, or both are required for this early PIF3 activity. Whereas the WT construct (HA:WT-PIF3) restored induction of *ELIP2* in the *pif3-3* background, the DNA binding-defective construct (HA:PIF3mbHLH) failed to complement *pif3-3* (Fig. 1*C* and SI Fig. 9*A*). In contrast, both the phyB-binding-defective mutant construct (HA:PIF3mAPB; Fig. 1*C* and SI Fig. 9*A*) and the phyA-binding-defective mutant construct (HA:PIF3mAPA; Fig. 1*D* and SI Fig. 9*B*) are comparable with the WT-PIF3 construct in their ability to complement *ELIP2* expression. Complementation by the HA:PIF3mAPA line was even observed after 1 h of FRc (Fig. 1*E* and SI Fig. 9*C*), where phyA is known to be the only active photoreceptor. We conclude that DNA binding but not individual phyA or phyB binding is required for PIF3-mediated *ELIP2* photoinduction.

However, in these singly defective mAPB and mAPA mutant lines, PIF3 still undergoes phy-induced phosphorylation, ubiquitination, and ultimately degradation by 1 h of Rc because of the redundancy of phyA and phyB action, leaving open the possibility that these processes could be required for the transcriptional activation function (11). We used a PIF3 derivative defective in binding to both phyA and phyB (YFP:PIF3mAPAmAPB) , which is defective in all phy-induced modifications (11), to test whether these functions are required for PIF3-dependent *ELIP2* induction. Importantly, the double mutant line was fully competent in restoring *ELIP2* induction (Fig. 1*F* and SI Fig. 9*D*), suggesting that neither direct interaction with either photoactivated phy molecule nor the resultant modifications are required for PIF3 transcriptional activity. Similar results were obtained for two other PIF3-regulated genes (7, 23), Chalcone synthase (*CHS*; AT5G13930) and photosystem II reaction center W subunit (*PSBW*; AT4G28660) (SI Fig. 10*A* and *B*), indicating that this unexpected mechanism of PIF3 involvement in light-induced *ELIP2* expression likely extends to other PIF3-dependent genes, including the presumed direct PIF3 target, *CHS* (23).

PIF3 Acts as a Constitutively Coactive Transcription Factor, with phy-Induced Degradation Acting to Limit This Early Activity. The above experiments imply that PIF3 acts as a constitutive coactivator in facilitating light-induced transcriptional activation rather than itself being a directly phy-activated transcription factor. To test this prediction rigorously, we determined whether PIF3 can activate transcription in the dark. We tested whether a Gal4 DNA-binding domain (GBD)-PIF3 fusion protein (GBD:PIF3) could activate a *luciferase* (*LUC*) reporter gene carrying upstream copies of the GBD-binding site after transient transfection into etiolated *Arabidopsis* hypocotyls, as described in ref. 15. GBD:PIF3 transactivates ≈3-fold compared with GBD alone without light induction in this context (Fig. 1*G*), a robust response because the strong activator GBD:AD positive control transactivates only 2-fold more in this system (Fig. 1*G*).

The fact that activation by the GBD:PIF3 fusion protein was stronger in the dark than in the light (Fig. 1*G*) suggested the possibility that phy-induced degradation of endogenous PIF3 during initial light exposure may contribute to the shutoff of

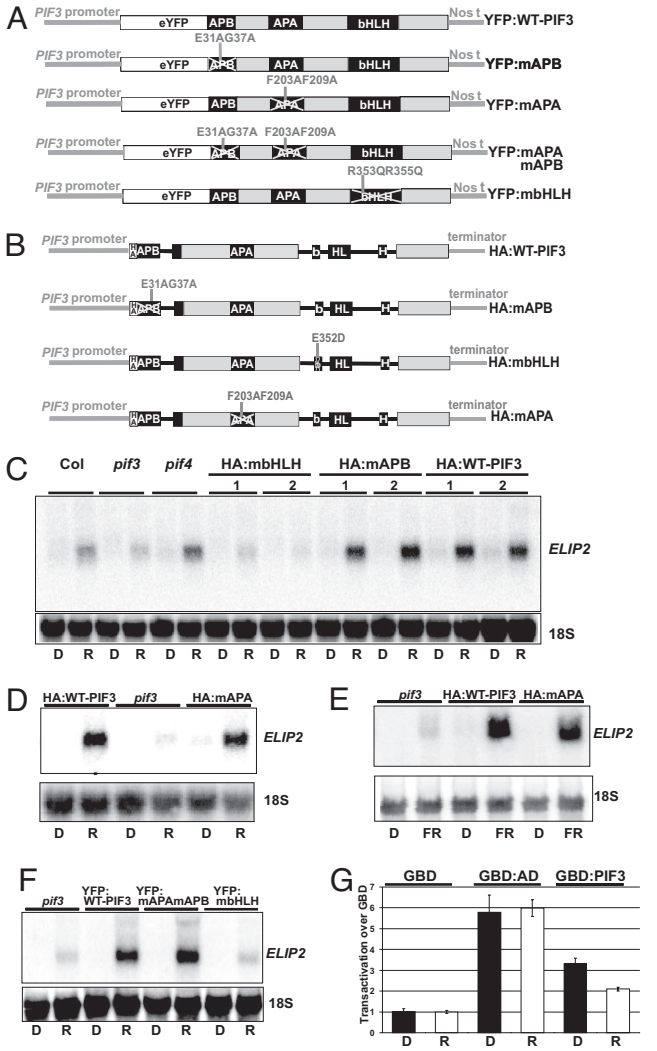

Fig. 1. PIF3 acts as a constitutive transcription factor in *ELIP2* induction independently of phyA or phyB interaction but requiring DNA association. (*A*) Schematics and terminology for pif3::YFP:PIF3 transgenic *pif3-3* rescue constructs. (*B*) Schematics and terminology for genomic HA:PIF3 transgenic *pif3-3* rescue constructs. (*C*) *ELIP2* induction at 1 h requires PIF3 DNA but not phyB binding. Four-day dark-grown seedlings were maintained in the dark (D) or exposed for 1 h to Rc at 9 μmol m^{-2} s^{-1} (R). Total RNA was extracted and probed for *ELIP2* and rehybridized for 18S rRNA as a loading control. HA:WT-PIF3 caused overexpression of ELIP2 at 1 h, consistent with the higher PIF3 levels in these lines compared with Col WT seedlings (SI Fig. 7). The numbers 1 and 2 refer to independent transgenic lines 1 and 2. (*D*) *ELIP2* induction at 1 h Rc does not require binding of PIF3 to phyA. Treatments were as in *C*. (*E*) *ELIP2* induction at 1 h FRc does not require binding of PIF3 to phyA. Treatments were as in *C*, except that FR seedlings were exposed for 1 h to FRc at 15 μmol m^{-2} s^{-1}. (*F*) *ELIP2* induction at 1 h Rc does not require PIF3 binding to phyA or phyB. Treatments of Col, *pif3*, YFP:WT-PIF3, or YFP:PIF3mAPAmAPB seedlings were as in *C*. For quantification of blots in *C–F* see SI Fig. 9*A*. (*G*) PIF3 is a transcriptional activator with maximal activity in darkness. Four-day-old dark-grown Col WT seedlings were bombarded with effector constructs expressing a GBD:PIF3 fusion (GBD:PIF3), GBD:AD fusion, or GBD alone. Seedlings were treated for 15 min with FR light and then kept in darkness for further 18 h (D) or treated every 2 h with 5-min R light pulses (R). Each column represents the mean of four biological replicates, error bars denote SE. Fold activation represents fold increase in the photon count of the firefly versus *Renilla* luciferases of GBD effector alone, which is set to 1. GBD, Gal4 DNA-binding domain; AD, Gal4 activation domain. Schematics of constructs used here can be found in SI Fig. 15.

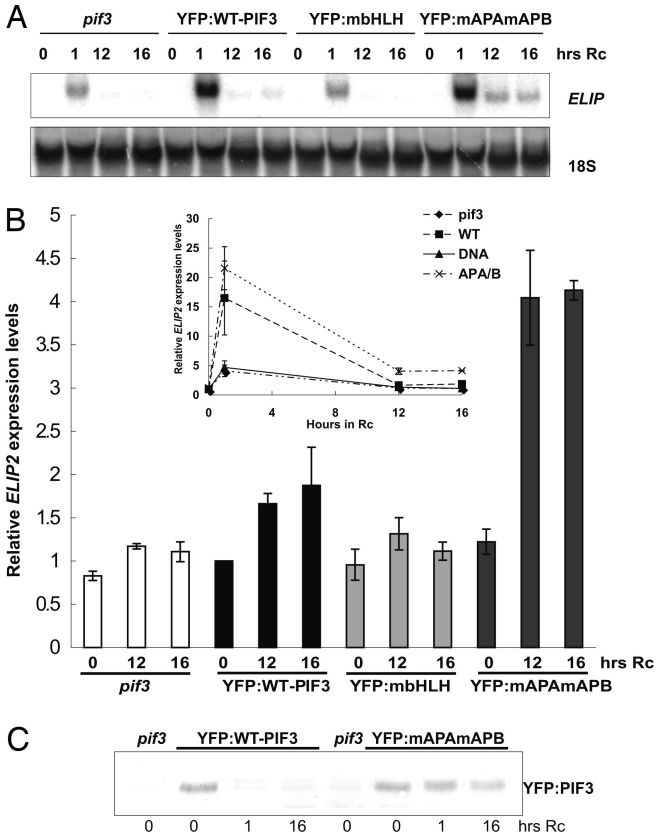

Fig. 2. phy-induced degradation of PIF3 acts as a timing gate on PIF3 constitutive transcriptional activity. (*A*) *ELIP2* mRNA persists after induction peak in prolonged Rc in YFP:PIF3mAPAmAPB lines. Four-day-old dark-grown Col, *pif3*, or YFP:WT-PIF3, YFP:PIF3mbHLH, or YFP:PIF3mAPAmAPB seedlings were maintained in dark (0) or exposed to Rc at 9 μmol m^{-2} s^{-1} for 1, 12, or 16 h. RNA processing was as in Fig. 1 *C–F*. (*B*) Quantification of *ELIP2* mRNA levels under prolonged Rc. Results shown are from triplicate RNA blot data as in *A*. Error bars represent SE. (*C*) YFP:PIF3mAPAmAPB protein levels persist in prolonged Rc. Four-day-old dark-grown *pif3*, YFP:WT-PIF3, or YFP:PIF3mAPAmAPB seedlings were kept in darkness (0) or exposed to Rc at 9 μmol m^{-2} s^{-1} for 1 or 16 h. Direct protein extracts were probed for YFP:PIF3 fusion proteins by using purified PIF3 antiserum.

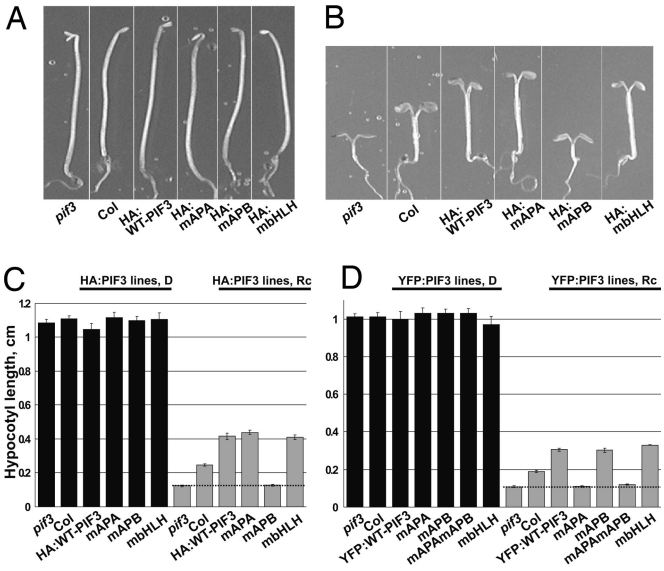

Fig. 3. PIF3 interaction with phyB but not with DNA is required for control of cell elongation responses under long-term Rc irradiation. (*A*) Visible phenotype of 4-day dark-grown genomic-HA:PIF3 transgenic seedlings. Shown are Col wild-type (Col), parental *pif3-3* mutant, and one representative transgenic line for each construct (see Fig. 1*B*) (additional independent transgenic lines are shown in SI Fig. 12). Col denotes the parental wild type of *pif3*. (*B*) Visible seedling phenotypes of the same lines as in *A* grown for 4 days in Rc at 9 μmol m^{-2} s^{-1}. (*C*) Hypocotyl lengths of genomic-HA:PIF3 transgenic seedlings after 4-day growth in darkness or Rc at 9 μmol m^{-2} s^{-1}. Data are for the same genotypes as in *A*. The horizontal dotted line denotes hypocotyl length of the *pif3-3* parental line. Error bars denote SE. (*D*) Hypocotyl lengths of PIF3::YFP:PIF3 transgenic seedlings. Growth and light treatments were as in *C*. Data are for the Col wild-type, parental *pif3-3* mutant, and one representative transgenic line for each construct (see Fig. 1*A*) (additional independent transgenic lines are shown in SI Fig. 12). The horizontal dotted line denotes hypocotyl length of the *pif3-3* parental line. Error bars denote SE.

ELIP2 transcription. Previous studies indicated that *ELIP2* transcript levels increase rapidly after exposure to Rc, reach maximal levels between 1 and 3 h, decline significantly by 6 h, and reach dark levels by 12 h, with no further increase at longer time points (6, 7). We tried to determine whether PIF3 degradation participated in shaping this transient induction profile by comparing the 16-h time course of *ELIP2* expression in YFP:WT-PIF3 and YFP:PIF3mAPAmAPB lines, the latter expressing constitutively high levels of the PIF3 fusion protein (11). Induction of *ELIP2* is similar for both lines at 1 h, but the YFP:PIF3mAPAmAPB line exhibited 2-fold more induction than YFP:WT-PIF3 at 12 and 16 h (Fig. 2*A* and *B*), indicating that shutoff is slower in the mutant lines. However, shutoff still occurs, even though YFP:PIF3mAPAmAPB protein levels do not decrease significantly (Fig. 2*C* and SI Fig. 11), indicating that other factors, in addition to PIF3, must participate in the shutoff reaction (see *Discussion*). We note that, like the genomic rescue lines, the YFP:PIF3mbHLH lines also cannot reinstate *ELIP2* induction levels comparable with YFP:WT-PIF3 (Fig. 2*A*).

Taken together, these studies indicate that during initial light exposure, PIF3 acts as a constitutively coactive transcription factor, meaning that its intrinsic transcriptional activation activity is light-independent but that on its own, in the context of its normal target promoters, PIF3 is not sufficient to achieve induction of phy-induced genes. Therefore, although PIF3 is necessary for the light induction of target genes, it most likely acts in conjunction with one or more additional, light-dependent, conditionally acting factor(s) to translate the phy signal into early light induction. Further, for rapidly and transiently induced, PIF3-dependent genes, like *ELIP2*, light-induced PIF3 degradation may serve as part of a timing gate regulating the temporal profile of *ELIP2* expression.

PIF3-Dependent Control of Hypocotyl Growth During Long-Term Rc Requires Association with phyB, but Not phyA or DNA. We analyzed the molecular activities of PIF3 necessary for it to negatively regulate light-imposed hypocotyl inhibition by assaying the ability of various HA:PIF3 transgenic constructs to rescue *pif3* mutant phenotypes after prolonged continuous R irradiation (Rc) (Fig. 3 *A–C* and SI Fig. 12*B*). All transgenics behaved normally in the dark, with no obvious differences in hypocotyl growth (Fig. 3 *A* and *C*). As expected, the WT-PIF3 line (HA:WT-PIF3) rescued the *pif3* mutant in Rc and showed a mild overexpression phenotype, displaying somewhat longer hypocotyls than WT (Col) seedlings (Fig. 3 *B* and *C*) (11). Mutant mAPA versions of HA:PIF3 also rescued similar to HA:WT-PIF3. Mutant mAPB HA:PIF3 and YFP:PIF3 versions, however, both failed to rescue *pif3* (Fig. 3 C and D), and double mutated mAPAmAPB (asYFP:PIF3 constructs) was also unable to rescue (Fig. 3*D*), even though this mutant protein can accumulate to higher levels than WT-PIF3 (Fig. 2*C* and SI Fig. 11). These data indicate that the effect of PIF3 on hypocotyl

growth under long-term Rc is strictly dependent on the ability of PIF3 to interact with phyB and independent of elevated levels of noninteractive PIF3. That physical association of PIF3 with phyB but not phyA is required for growth promotion is consistent with the fact that hypocotyl growth is primarily controlled by phyB, with phyA playing only a minor, if any, role (24). In sharp contrast, the behavior of the lines defective in DNA binding was quite surprising. The HA:PIF3mbHLH construct rescues *pif3*, restoring a phenotype indistinguishable from that produced by WT-PIF3 (Fig. 3*A–C*), suggesting that the DNA-binding feature of PIF3 is not required for its role in modulating hypocotyl growth. Similar data for all lines were obtained for the YFP:PIF3 set of transgenic lines (Fig. 3*D* and SI Fig. 12*A*).

PIF3 Controls Growth Responses in Long-Term Rc by Modulating phyB Photoreceptor Levels. How might PIF3 regulate the hypocotyl growth response? Because this response is sensitive to relatively small changes in the absolute amounts of the phyB photoreceptor (20, 21, 25), one simple possibility is that PIF3 might regulate phyB abundance (7). We used anti-phyB monoclonal antibodies to perform carefully quantitated Western blot analysis on WT (Col), *pif3* mutant, and HA:PIF3 rescue lines. Visual analysis indicates that the amount of phyB protein after 4 days in Rc is inversely related to the amount of phyB-interactive PIF3: (*pif3* = PIF3mAPB > Col > HA:WT-PIF3; Fig. 4*A* and SI Fig. 13). We quantified this effect by measuring the amount of phyB signal in three independent experiments. Consistent with the qualitative results, both the *pif3* mutant and the PIF3mAPB rescue line had 1.5-fold more phyB than Col, whereas the WT-PIF3 rescue line had 2-fold less phyB than Col (Fig. 4*B*). Importantly, our internal control indicated that no differences in phyA levels were detectable between these four genotypes in response to the prolonged Rc irradiation (Fig. 4*B*). The Rc-induced differences in phyB levels are not evident in the first 3 h after exposure to Rc but become progressively more apparent after 6–24 h of Rc (Fig. 4*C*). These differences in phyB level do not result from differential transcription of *PHYB* because *PHYB* transcript levels are identical in the various lines (Fig. 4*D*). Thus, a posttranscriptional mechanism must regulate phyB levels.

One prediction of the conclusion that PIF3 acts through control of phyB abundance in long-term Rc is that a *phyBpif3* double mutant should have the same phenotype in long-term Rc as the *phyB* mutant. Because PIF3 regulates phyB levels, the removal of PIF3 from a mutant already lacking phyB should have no additional effect on the phenotype. The data show that this is indeed the case (SI Fig. 14; see also ref. 26).

Discussion

Certain members of the PIF3 family of transcription factors, implicated in mediating phy signaling, such as PIF3 (7), PIF1/PIL5 (14, 15, 27), and PIF5 (16), are required for two temporally separated responses. After light activation, different family members appear to regulate different early responses. PIF3, for example, is required for expression of rapidly light-induced, nuclear-encoded, chloroplast-targeted gene products. During long-term Rc irradiation, PIFs negatively regulate a single visible morphogenic process, primarily controlled by the phyB photoreceptor. It has remained unclear how PIFs mediate both responses. By dissecting the domains required for each process, we have discovered that temporal distinction is achieved by using distinct molecular functions of PIF3. The principal conclusion of this work is to suggest the unrecognized possibility that PIF3 exhibits mechanistic duality. Immediately after light activation, PIF3 functions as a transcription factor, whereas over long-term continuous irradiation, PIF3 tunes morphological output by regulating phyB photoreceptor level posttranscriptionally (Fig. 5). Below, we discuss the evidence for and implications of these findings.

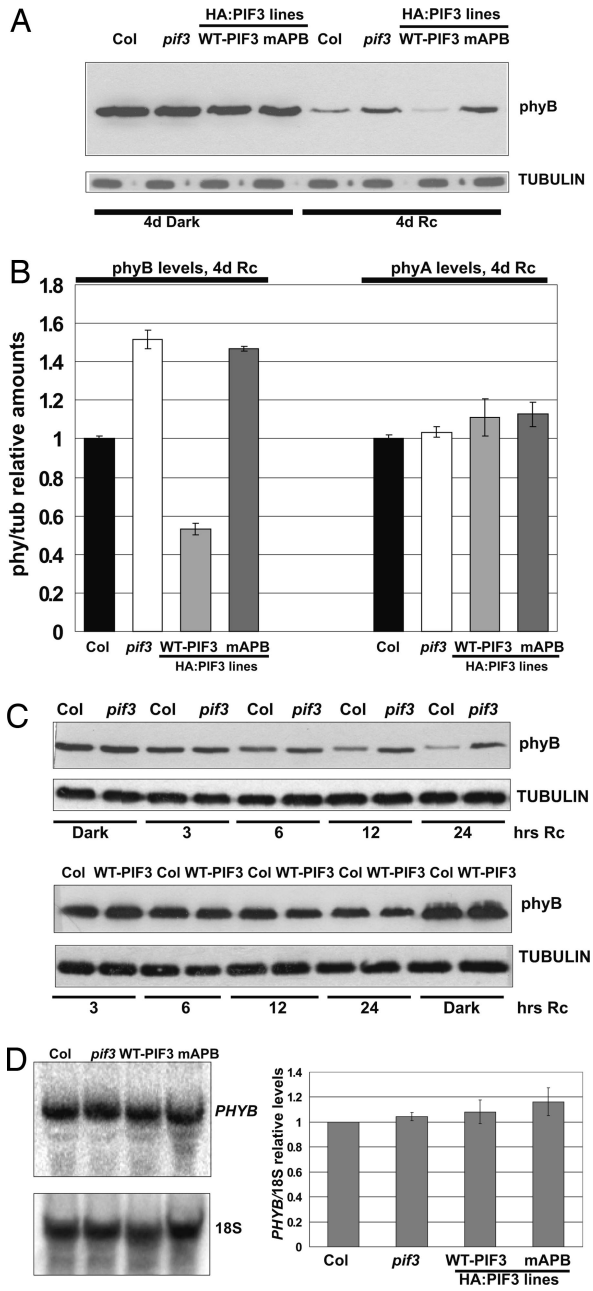

Fig. 4. Under long-term, Rc, PIF3 acts to regulate phyB protein levels in an APB-dependent manner. (*A*) PIF3 affects phyB levels in 4-day Rc-grown plants through the APB. *pif3*, Col, HA:WT-PIF3, or HA:PIF3mAPB seedlings were grown either 4 days in darkness (4d Dark) or 4 days under Rc at 9 μmol m^{-2} s^{-1} (4d Rc). Direct extracts were probed for phyB protein or α-tubulin with monoclonal antibodies. (*B*) Quantification of differences in phyA and phyB levels of 4-day, Rc-grown seedlings. Three independent lots of *pif3*, Col, HA:WT-PIF3, or HA:PIF3mAPB seedlings were grown, and protein extracts were probed for phyA or phyB and tubulin as a loading control. To ensure linearity of phyA, phyB, and tubulin immunoblot signals, a 2-fold extract dilution curve was run and exposed in parallel to the three replicates. Error bars represent SE. (*C*) Effect of PIF3 on phyB protein levels occurs late after exposure to Rc. *pif3*, Col, and HA:WT-PIF3 (WT-PIF3) seedlings grown 4 days in darkness were maintained in the dark (0) or exposed to 3, 6, 12, or 24 h of Rc at 9 μmol m^{-2} s^{-1}. Protein extraction and phyB Western blotting were as in *A*. (*D*) PIF3 does not affect *PHYB* transcript levels of 4-day, Rc-grown seedlings. *pif3*, Col, HA:WT-PIF3, and HA:PIF3mAPB seedlings were grown for 4 days in Rc as above. Total RNA was extracted, blotted, and probed for *PHYB* or 18S rRNA as a loading control. (*Left*) One representative blot. (*Right*) Quantification of three independent replicates.

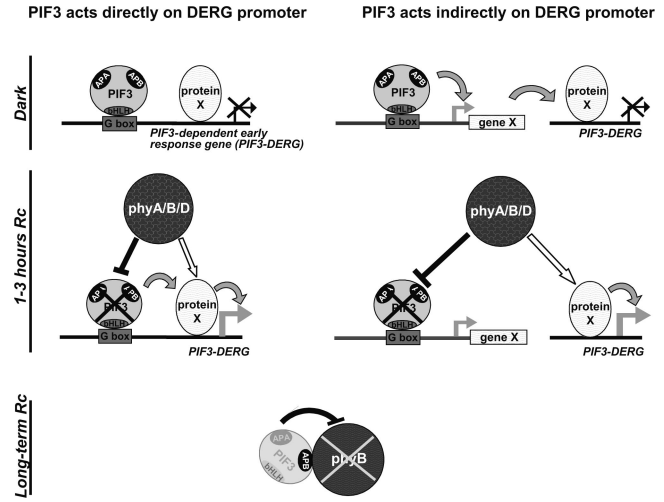

Fig. 5. Model for contrasting PIF3 actions in early light-induced gene expression and long-term growth responses. In darkness, PIF3 is either resident together with protein X at a single *PIF3*-dependent early-response gene (*PIF3-DERG*) promoter, like *ELIP2* (*Left*) or at the promoter of the *PROTEIN X* gene (*Right*). PIF3 and protein X are both required for the induction of *PIF3-DERG* by light, indicating that they both act positively in this process. PIF3 acts constitutively, requiring its bHLH domain. After light exposure, phys concomitantly both activate protein X transcriptional activity and induce PIF3 phosphorylation and degradation via the PIF3 APA and APB motifs, in the time window when *PIF3-DERG* is being induced. This action results in a transient *PIF3-DERG* induction profile. Under long-term irradiation conditions, the low steady-state levels of PIF3 are insufficient for maintaining high *PIF3-DERG* expression but act now, postranslationally, to induce phyB degradation, in a process requiring the PIF3 APB for direct association with the photoreceptor. This mechanism thus involves indirect global control of phyB output, in this case, hypocotyl growth.

Previous hypotheses about PIF3 action in light-mediated gene expression have proposed that the protein is either a light-inactivated transcriptional repressor (22) or a light-activated transcriptional activator (9). In both scenarios, the nuclear PIF3 molecule (11, 12) is modified by nuclear-imported, phy-Pfr to affect either its light stability and/or transcriptional activation potential. The scenario that PIF3 acts as a repressor of light-induced, early-response genes in the dark and that its removal derepresses these genes is rendered unlikely by detailed microarray analysis, which indicates that the vast majority of PIF3-dependent, rapidly light-inducible genes do not exhibit any difference in expression between WT and *pif3* mutants in the dark (7). Fig. 1 in the present work also illustrates this behavior for *ELIP2*.

Here, we have presented three findings that together argue that PIF3 is also not a light-activated factor but instead is a constitutive transcriptional coactivator, which is co-opted in an unknown way to mediate expression immediately after light exposure. First, mutant rescue experiments indicate that early-response changes in gene expression require the DNA-binding capacity of PIF3 but not its capacity to bind either phyA or phyB (Fig. 1 *C–F*). Second, a PIF3 derivative defective in binding to both phyA and phyB is fully competent for activating rapid, light-induced gene expression (Fig. 1*F*). Because this variant does not exhibit phy-induced phosphorylation, ubiquitination, or degradation, the data argue against the proposal that these processes are necessary for activation. Although it remains possible that phosphorylation events undetected by the gel shift assay occur, it seems unlikely that they are involved in Rc light-induced transcriptional activation of PIF3 because this involvement would require phyC, D, or E mediation, and they do not induce *ELIP2* expression in the absence of phyA and phyB (6). Finally, the GBD:PIF3 fusion protein transactivates from a GBD-binding site in the dark, indicating that unmodified PIF3 is capable of gene activation in this context (Fig. 1*G*). Taken together, these experiments indicate that PIF3 is constitutively capable of activating transcription and is not a direct mediator of phy-induced transcriptional activation at target promoters.

Why then, does PIF3 require phys to mediate light-induced transcriptional activation at photoresponsive genes, such as *ELIP2*, *CHS*, or *PSBW*? The data suggest that a second factor or factors (factor X for convenience here) must be responsible for direct transduction of photoactivated phy signals, playing the role of "light-responsive switch." Two principal possibilities for factor X function can be envisioned, given the available data: factor X could be a coregulator at promoters of photoresponsive genes together with PIF3, or factor X could be constitutively transcribed under PIF3 control and itself function as the light-conditional positive regulator at the promoters of photoactivated genes (Fig. 5). The first possibility is consistent with the behavior of *CHS* because it is has been identified as a direct PIF3 target gene by ChIP analysis (23) but does not require direct phy/PIF3 interaction for its light induction, as shown here.

What is the role of phy-induced PIF3 degradation upon initial light exposure? PIF3 degradation may contribute to the transient induction profile of *ELIP2* and other rapidly photoactivated genes (6, 7). Because PIF3 degradation significantly precedes shutoff of *ELIP2*, PIF3 level cannot be the sole determinant of the kinetics of transcriptional shutoff. However, when PIF3 is maintained at an artificially high level by preventing its degradation, induction is prolonged (Fig. 2). Therefore, we suggest that PIF3 is one component of a timing gate for light-induced gene expression.

Collectively, the above considerations suggest a potentially general dual molecular mechanism of light-induced phy activity at target promoters (Fig. 5): (*i*) a negative channel involving temporally paced removal of a positively acting, intrinsically active transcriptional regulator or coregulator (such as PIF3); and (*ii*) a positive channel involving phy-induced activation of a positively acting, conditionally activatable transcriptional regulator or coregulator (such as factor X). It remains possible, however, that the biochemical mechanism of intermolecular signal transfer might be identical in each case. For example, light activation of factor X might involve phy-induced phosphorylation of the factor by direct interaction with the photoreceptor molecule, as demonstrated for PIF3 (11).

The following findings argue that PIF3 acts posttranscriptionally to fine-tune the amount of the phyB photoreceptor, thereby globally influencing seedling photomorphogenic responses. First, only the phyB-binding capability of PIF3 and not its ability to bind either DNA or phyA is required for this response. Second, the amount of phyB is inversely related to the amount of PIF3. Importantly, modulation of phyB abundance depends on direct binding of this photoreceptor molecule to PIF3 because PIF3mAPB mutants that cannot bind phyB exhibit no such phyB modulation (Fig. 4*A*). Finally, lines with different amounts of PIF3 exhibit comparable amounts of *PHYB* mRNA, arguing that regulation is not at the transcriptional level (Fig. 4*D*). Thus, PIF3 apparently antagonizes the long-term, light-regulated, phy-induced inhibition of hypocotyl elongation indirectly by negative-feedback modulation of phyB protein levels rather than participating directly as an intermediate in the phyB signaling pathway, as proposed (3, 7).

The fact that the visible, PIF3-dependent, hypocotyl phenotype is photoreceptor-specific, depending on direct binding of PIF3 to phyB, but not to phyA, is consistent with the established specific function of phyB in controlling hypocotyl inhibition in

Rc (19). These observed differences in phyB levels, although quantitatively modest, occur over a range where hypocotyl responsiveness to Rc is known to be highly sensitive to relatively small changes in absolute phyB levels (20, 21, 25). The molecular mechanism by which PIF3 might modulate phyB levels is unknown. The possibility that it involves codegradation of the interacting proteins might initially appear unlikely because (*i*) phyB levels are not affected in the first 3 h after light exposure, during which PIF3 is rapidly degraded ($t_{1/2}$ ≈15 min) to a new lower steady-state (7, 11–13) (Fig. 4*C*), and (*ii*) phyA protein levels do not appear to be affected by the presence of PIF3, even though this photoreceptor, analogously to phyB, interacts with PIF3 and induces PIF3 phosphorylation and degradation. Regardless of the mechanism, however, in the long-term, the low-abundance, steady-state levels of PIF3 in continuous light (11) could function to fine-tune phyB levels. In fact, the possibility that other phyB-interacting PIFs act additively in the same manner as PIF3 to regulate phyB levels under long-term Rc is currently emerging from studies on other members of the bHLH subfamily 15 (16, 28).

The duality of molecular function of PIF3 detailed above could provide a new general mechanism of action for other PIF3-related PIF transcription factors: PIFs may both help the emerging deetiolating seedling to rapidly initiate the photomorphogenic program in the first hours after light exposure, through a cascade of transcriptional events induced by phyA and phyB (4, 6) and later assist the fully deetiolated seedling to optimize its long-term growth responses to the prevailing light environment by modulating the signaling output from phyB, the dominant receptor under these conditions.

Materials and Methods

Plants. Transgenic *pif3*::*YFP*:*PIF3* WT-PIF3, mAPA, mAPB, and mAPAmAPB lines were as described in ref. 11. For the YFP:PIF3mbHLH lines, R353Q/R355Q mutations were introduced into the *pif3*::*YFP*:*PIF3* construct, and *pif3-3* plants were transformed as described in ref. 11. For the genomic HA:PIF3 lines, the genomic *PIF3* locus comprising 2,000 bp upstream of the initiating ATG and 500 bp downstream of the stop codon was PCR-amplified, including a single N-terminal HA tag, from Col-O genomic DNA and cloned into pZP121. Site-directed mutagenesis and transformation of *pif3-3* were performed as described in ref. 11. For hypocotyl measurements, plants were germinated as described in ref. 7 and grown for 4 days either in darkness or Rc at 9 μmol m^{-2} s^{-1}.

Western Blotting. Seedling growth, light treatments, and direct protein extractions were as described in ref. 11. For phyA and phyB blots, 1% SDS was used instead of 5%, and 40 mM 2-mercaptoethanol was replaced with 50 mM metabisulfite. Protein concentrations were determined with a Dc kit (Bio-Rad), and 2-mercaptoethanol was added just before loading. For detailed phyA and phyB level quantification, see *SI Text*.

Transient Transfection Assays. Transient transfections, light treatments, and luciferase assays were as described in ref. 15. For GBD:PIF3, the PIF3 ORF was PCR- amplified and cloned into pMN6 in-frame with GBD.

Northern Blot Analysis. RNA analysis was performed as described in ref. 7. For *PHYB* blots, a probe consisting of the last 442 bp of the coding sequence was radiolabeled, hybridized, and normalized with 18S rRNA.

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